AD_____

Award Number: W81XWH-06-1-0129

TITLE: Aberrant NPM Expression during Nf1Loss and Its Role in Promoting Proliferation

PRINCIPAL INVESTIGATOR: Jason Dean Weber, Ph.D.

CONTRACTING ORGANIZATION: Washington University
St. Louis, MO 63110

REPORT DATE: December 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-12-2006	Final	15 Nov 2005 – 14 Nov 2006

4. TITLE AND SUBTITLE

Aberrant NPM Expression during Nf1Loss and Its Role in Promoting Proliferation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0129

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jason Dean Weber, Ph.D.

Email: jweber@im.wustl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Washington University
St. Louis, MO 63110

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Nf1 encodes a novel tumor suppressor protein named neurofibromin that acts to inhibit the Ras oncogene. A recent study demonstrated that the nucleolar proto-oncogene nucleophosmin (NPM) was highly expressed in Nf1deficient astrocytes along with numerous other ribosomal biosynthesis proteins. NPM senses abnormal growth signals within the astrocyte and responds by increasing protein synthesis rates. We are hypothesizing that these increased rates are partly responsible for the aberrant growth of Nf1-deficient astrocytes. Thus, the goal of this work will be t0 determine the contribution of elevated NPM levels to astrocyte proliferation. We believe that the insights gained from this study will greatly improve our understanding of the mechanism (s) by which neurofibromin loss promotes cell growth and proliferation. This work could be translated into any number of cell systems implicated in NF1 due to its mechanistic and evolutionary conservation. In addition, our results will open up a new generation of targets aimed at disrupting aberrant protein translation networks that appear to go awry during Neurofibromatosis.

15. SUBJECT TERMS
Neurofibromatosis, protein translation, NF1, neurofibromin, astrocytes

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	46	USAMRMC

Table of Contents

INTRODUCTION

Pilocytic astrocytomas are the second most common tumor seen in individuals with Neurofibromatosis Type 1 (NF1) with approximately 15% of children affected by NF1 exhibiting optic pathway gliomas[1-3]. As previously shown for other tumor pre-disposition syndromes, individuals with NF1 begin life with one mutated and one functional copy of the *Nf1* gene. *Nf1* encodes a novel tumor suppressor protein named neurofibromin that acts as a GTPase activating protein to functionally inactivate Ras molecules[4-7]. While much has been learned about how neurofibromin inactivates Ras, only recently has effort been spent dissecting the numerous downstream signaling pathways affected by *Nf1* loss. Specifically, little is known about the role of neurofibromin in regulating cell growth, increases in protein production, and how this might effectively manipulate astrocyte proliferation. A recent study demonstrated that nucleophosmin (NPM) was highly expressed in *Nf1*-deficient astrocytes along with numerous other ribosomal biosynthesis proteins[8]. NPM is a nucleolar proto-oncogene whose expression is tightly regulated by growth factor signals[9,10]. Additionally, NPM mRNA contains a signature 5'-terminal oligo-pyrimidine tract that responds to S6K signals for its active translation, placing NPM downstream of the mTOR/S6K cascade[11]. Increasing efforts have been placed on identifying the biological role of NPM in promoting cell growth and proliferation. To this end, several groups have reported that NPM is the target of the nucleolar ARF tumor suppressor, underscoring the importance of NPM in promoting cell growth[12-14]. ARF inhibits both the ribosome processing and nuclear export functions of NPM to induce proliferative arrest, providing insights into the critical growth-promoting functions of NPM[12,13]. Nuclear export of NPM requires a conserved Crm1 motif and, although we have not published these preliminary data, mutation of two leucine residues to alanines at positions 42 and 44 results in the nuclear retention of NPM. We propose to evaluate the role of aberrant S6K/NPM activities on *Nf1*-null astrocyte growth and proliferation.

The purpose of this study is demonstrate the impact of aberrant NPM expression on astrocytes lacking *Nf1* in order to more completely understand the growth and proliferative advantages bestowed upon these cells. The specific aims of this project are to 1) determine whether loss of *Nf1* results in NPM-directed increases in ribosome production and 2) examine the sensitivity of NPM-overexpressing *Nf1*-null astrocytes to S6K inhibition. Since NPM has been shown to be overexpressed in the absence of neurofibromin, we propose that increases in NPM will augment ribosome biogenesis. We have generated a dominant negative NPM mutant that suppresses nuclear export of the ribosome in wild-type mouse embryonic fibroblasts. Using this novel export inhibitor, we will determine whether NPM directs nuclear export of ribosomes in astrocytes. Additionally, we will determine the contribution of hyperactivated S6K to increases in NPM expression, ribosome export and growth in *Nf1*-deficient astrocytes. This could reveal a novel therapeutic pathway responsible for regulating *Nf1*-null cell growth and proliferation.

We have obtained *Nf1*-null astrocytes and have used these cells for in vitro manipulations involving retroviral infection, Nucleofector™ transduction, cell growth and proliferation assays, and Western and Northern blot analysis. We have frequently performed all of the assays outlined in the Statement of Work including ribosomal RNA export, labeling of

newly synthesized ribosomal RNAs, S6K activity assays, BrdU incorporation into replicating DNA, flow cytometry for DNA analysis, and proliferation assays. We are in possession of the NPM export mutant as well as the dominant negative S6K construct. We believe that the insights gained from this study will greatly improve our understanding of the mechanism(s) by which neurofibromin loss promotes cell growth and proliferation. This work, although performed in primary astrocytes, could be translated into any number of cell systems implicated in NF1 due to its mechanistic and evolutionary conservation. In addition, our results will open up a new generation of targets aimed at disrupting aberrant protein translation networks that appear to go awry during Neurofibromatosis.

BODY

Neurofibromatosis type 1 (NF1) is a common autosomal dominant tumor predisposition syndrome in which affected individuals develop astrocytic brain tumors (gliomas). To determine how the *NF1* gene product (neurofibromin) regulates astrocyte growth and motility relevant to glioma formation, we have employed *Nf1*-deficient primary murine astrocytes. *Nf1*-/- astrocytes exhibit increased protein translation and cell proliferation[8], which are mediated by Ras-dependent hyperactivation of the mammalian target of rapamycin (mTOR) protein, a serine/threonine protein kinase that regulates ribosomal biogenesis, protein translation, actin cytoskeleton dynamics, and cell proliferation. In this study, we demonstrate that *Nf1*-deficient astrocytes have fewer actin stress fibers and exhibit increased cell motility compared to wild-type astrocytes, which are rescued by pharmacologic and genetic mTOR inhibition. We further show that mTOR-dependent regulation of actin stress fiber formation, motility, and proliferation requires rapamycin-sensitive activation of the Rac1 GTPase, but not 4EBP1/S6K. *Nf1*-/- astrocytes also exhibit increased protein translation and ribosomal biogenesis through increased expression of the nucleophosmin (NPM) nuclear-cytoplasmic shuttling protein. We found that NPM expression in *Nf1*-/- astrocytes was blocked by rapamycin *in vitro* and *in vivo*, and that expression of a dominant-negative NPM mutant protein in *Nf1-/-* astrocytes rescued actin stress fiber formation and restored cell motility and proliferation to wild-type levels. Together, these data demonstrate that neurofibromin regulates actin cytoskeleton dynamics and cell proliferation through an mTOR/Rac1-dependent signaling pathway and identify NPM as a critical mTOR effector mediating these biologic properties in *Nf1*-deficient astrocytes. This data will be discussed in detail below.

First, we demonstrate that primary astrocytes lacking Nf1 exhibit enhanced cytoplasmic polysomes, ribosomes actively translating mRNAs (Figure 1). This experiment was performed exactly as outlined in our Statement of Work, Task 1. Astrocytes from wild-type and Nf1-deficient mice were plated in equal numbers, lysed and separated over sucrose gradients. The gradient was then analyzed by constant UV monitoring to determine where the rRNA fractions separated. A trace of this analysis is shown in Figure 1 and allows us to accurately measure the relative amounts of cytosolic ribosomes for each setting.

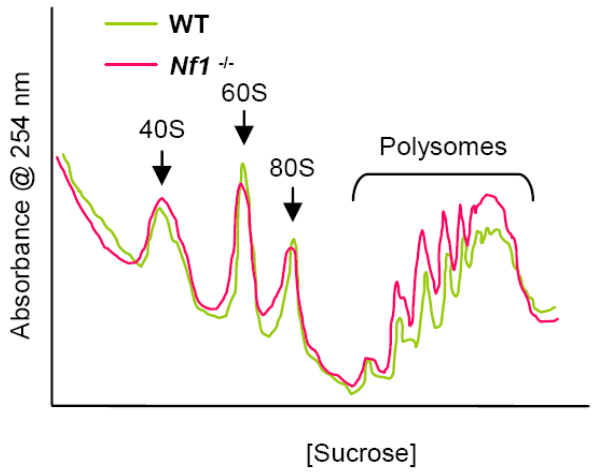


Figure 1. *Nf1*-/- astrocytes exhibit greater cytosolic polysomes. *Nf1*-/- astrocytes exhibit a 45% increase in polysome content as determined by ribosome fractionation over sucrose gradients.

Second, in order to answer the question proposed in the second part of Task 1, we determined that the increase in polysome formation in *Nf1*-deficient cells required functional NPM. Overexpression of *Nf1*-null astrocytes with a shuttling defective, dominant negative NPM mutant (NPMdL) resulted in a nearly complete inhibition of polysome content (Figure 2).

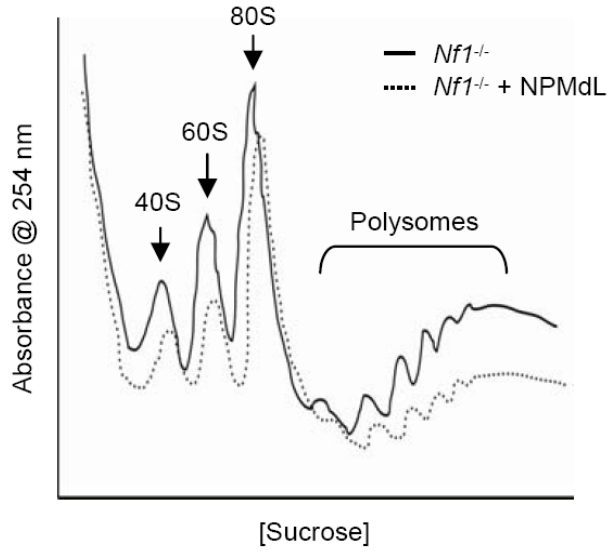


Figure 2. *Nf1*-/- astrocytes require NPM function for enhanced polysomes. *Nf1*-/- astrocytes transduced with NPMdL mutant were unable to maintain increased numbers of polysomes.

The final experiment outlined in Task 1 of our Statement of Work involved understanding the contribution of NPM function to the enhanced proliferation of *Nf1*-deficient astrocytes. In order to answer this extremely important question, we again transduced astrocytes with the NPMdL dominant negative mutant. Similar to the inhibition of polysome formation in *Nf1*-/- astrocytes transduced with NPMdL, expression of the NPMdL mutant also inhibited proliferation of *Nf1*-null astrocytes (Figure 3), suggesting that NPM nuclear export functions are absolutely required for enhanced *Nf1*-/- astrocyte proliferation. These results completed our analysis of ribosome content in cells lacking neurofibromin and also completed all of the experiments described in Task 1 of the Statement of Work.

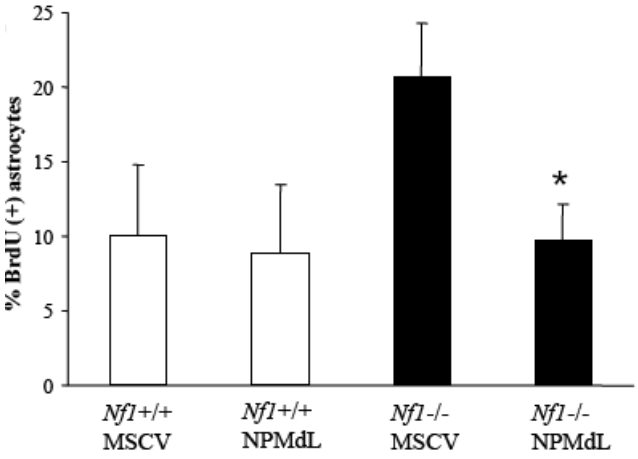


Figure 3. NPM mediates cell proliferation in *Nf1*-/- astrocytes. NPM function was inhibited genetically using a mutant protein (NPMdL) that can not shuttle between the nucleus and cytoplasm. Inhibition of NPM shuttling activity in *Nf1-/-* astrocytes restored cell proliferation to wild-type levels. Inhibition of NPM shuttling function had no effect on proliferation in wild-type cells. *, $p < 0.05$

For Task 2 of our Statement of Work, we had hypothesized that S6K1 acted downstream of mTOR to induce NPM expression and subsequent increases in polysome content in the absence of neurofibromin. However, our initial experiments provided evidence that S6K1 was not the upstream regulator of NPM. First, we determined that NPM expression in vivo was sensitive to rapamycin (Figure 4), indicating that mTOR is an upstream activator of NPM expression.

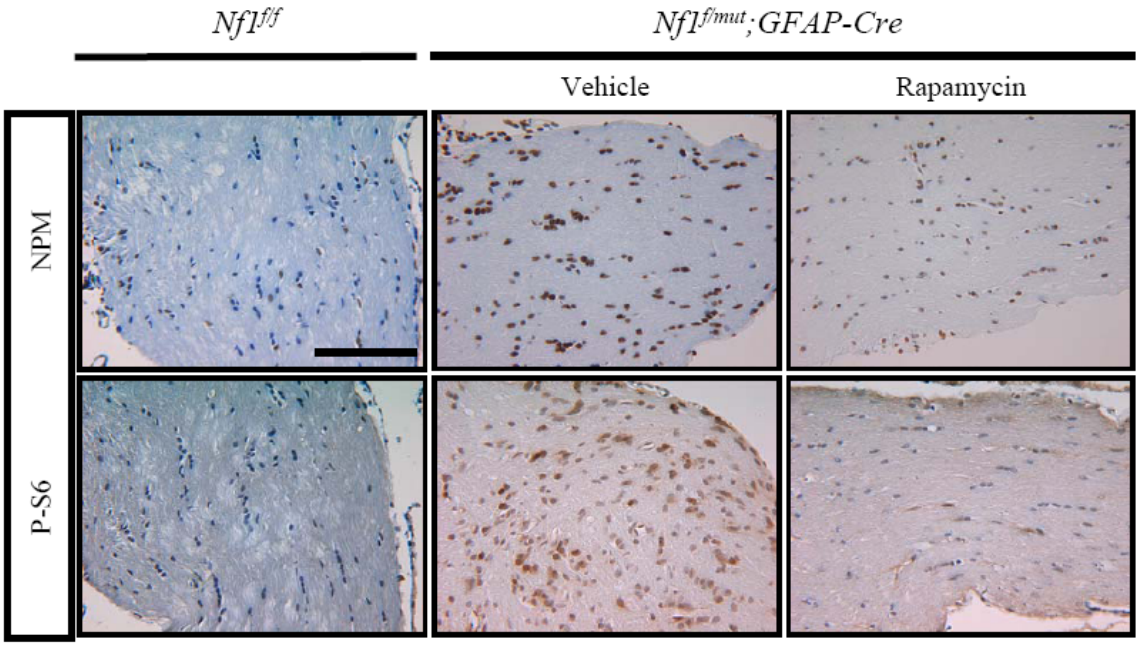


Figure 4. Nucleophosmin expression is regulated by mTOR signaling *in vivo*. Phospho-S6 and NPM are expressed at low levels in wild-type murine optic nerve (*Nf1f/f*), but are dramatically increased in a murine model of optic glioma (*Nf1f/mut;GFAP*-Cre), as shown by immunohistochemistry. Following rapamycin treatment *in vivo*, both NPM and P-S6 expression are decreased in the mouse optic gliomas. Scale bar=200 μm (10X).

Second, using a retroviral transduction system, we set out to determine the contribution of S6K1 to NPM expression in astrocytes. We reasoned that by overexpressing activated S6K1 in wild-type astrocytes, we should be able to push NPM expression to levels seen in *Nf1*-deficient astrocytes. This would show that S6K1 was the critical upstream regulator of NPM expression in astrocytes and could provide a novel target for inhibition in *Nf1*-deficient astrocytes. However, overexpression of S6K1 in wild-type astrocytes did not result in an increase in NPM protein expression (Figure 5A) even though it was still capable of phosphorylating S6.

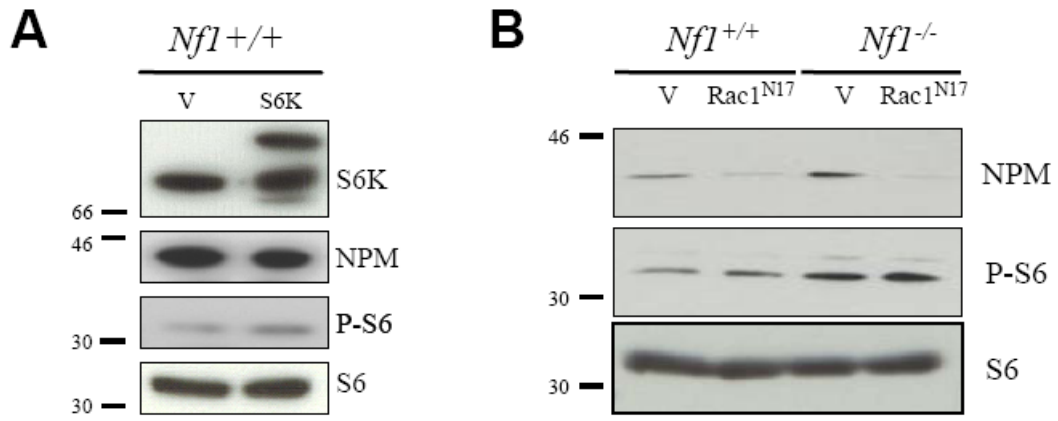


Figure 5. NPM expression is regulated by Rac1$_{N17}$, but not S6K, in *Nf1-/-* astrocytes. (A) Overexpression of the mTOR downstream target S6K in wild-type astrocytes did not result in an increase in NPM expression. Phosphorylation of the S6K target ribosomal S6, but not total S6 protein, was increased in astrocytes overexpressing S6K. (B) Expression of Rac1$_{N17}$ in *Nf1-/-* astrocytes decreases NPM expression, but does not attenuate S6 phosphorylation.

Even though this answered one of our major questions for Task 2, we could not move forward in our analysis of S6K1 as previously proposed, because it simply had no effect on NPM expression in astrocytes. This is strike contrast to its ability to regulate NPM expression in fibroblasts[14]. So we took a new approach to completing experiments for Task 2. Rac1 is a common target of the mTORC2 complex and we had generated preliminary data with Dr. David Gutmann suggesting that actin cytoskeletal changes (see attached paper) were regulated by loss of *Nf1* and increased NPM expression. Many of these changes can be attributed to Rac1 given its ability to regulate the actin cytoskeleton. We also hypothesized that Rac1 might also regulate NPM expression in cells lacking *Nf1*. Indeed, *Nf1*-/- astrocytes transduced with a dominant negative Rac1 mutant (Rac1^{N17}) displayed severe attenuation in NPM protein expression (Figure 5B), suggesting that Rac1 is responsible for regulating NPM expression in cells lacking *Nf1*.

Additional data and findings can be found in the appendices. Overall our findings point to NPM as a novel mediator of pro-growth, -proliferation, -motility, and –cytoskeleton changes in astrocytes lacking *Nf1*. These findings should provide a unique target in NPM for future studies into the therapeutic potential of targeting NPM function in Neurofibromatosis.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of NPM as a neurofibromin target
- Characterization of NPM as a required protein for the phenotypes observed in Neurofibromatosis
- Identification of rapamycin as a potential therapeutic agent for Neurofibromatosis

REPORTABLE OUTCOMES

- Sandsmark, D.K., Zhang, H., Hegedus, B., Pelletier, C.L., **Weber, J.D.** and Gutmann, D.H. (2007). Nucleophosmin mediates mammalian target of rapamycin-dependent actin cytoskeleton dynamics and proliferation in neurofibromin-deficient astrocytes. In press, *Cancer Research*.
- Scheidenhelm, D.K., Pelletier, C.L., **Weber, J.D**. and Gutmann, D.H. (2007). Mammalian target of rapamycin: master regulator of cell growth in the nervous system. In press, *Histology and Histopathology*.
- Patent Application: No. 11/478,474 for Methods for Treating Neurofibromatosis

CONCLUSIONS

This proposal was designed to determine the role of NPM in the proliferation of *Nf1*-/- astrocytes. To this end, we now provide evidence that NPM protein expression is elevated in *Nf1*-/- cells and that this up-regulation is completely abrogated by rapamycin treatment. Interestingly, induction of NPM through *Nf1* loss was not regulated by S6K but rather by Rac signal transduction. This is in contrast to NPM pathway regulation in fibroblasts where NPM protein induction is completely dependent on S6K activities[14]. Additionally, we now know that NPM regulates cytoskeletal changes in *Nf1*-deficient astrocytes. While we are not yet certain as to whether these effects are directly regulated by NPM proteins, we have now shown that nucleocytoplasmic shuttling of NPM is required for all of the biological effects of NPM (growth, proliferation and cytoskeletal changes) on *Nf1*-/- astrocytes. Taken together, our data now provides a compelling foundation for the further analysis of NPM as a potentially new therapeutic target in Neurofibromatosis.

REFERENCES

1. Listernick R,Darling C, Greenwald M, Strauss L, and Charrow J (1995) Optic pathway tumors in children. *Journal of Pediatrics* **127**, 718-722.
2. Listernick R, Louis DN, Packer RJ, and Gutmann DH (1997) Optic pathway gliomas in children with neurofibromatosis 1. *Annals of. Neurol*ology **41**, 143-149.

3. Listernick R, CHarrow J, Greenwald MJ, and Esterly NB (1989) Optic pathway gliomas in children with neurofibromatosis type 1. *Journal of Pediatrics* **114**, 788-792.
4. Xu G, Lin B, Tanaka K, Dunn D, Wood D, Gesteland R, White R, Weiss R, and Tamanoi F (1990) The catalytic domain of the neurofibromatosis type 1 gene product stimulates Ras GTPase and complements ira mutants of S. cerevisiae. *Cell* **63**, 835-841.
5. Xu G, O'Connell P, Viskochil D, Cawthon R, Robertson M, Culver M, Dunn D, Stevens J, Gesteland R, White R, Tamanoi F (1990) The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell* **62**, 599-608.
6. Martin GA, Viskochil D, Bollag G, McCabe PC, Crosier WJ, Haubruck H, Conroy L, Clark R, O'Connell P, Cawthon RM et al. (1990) The GAP-related domain of the neurofibromatosis type 1 gene product interacts with Ras p21. *Cell* **63**, 843-849.
7. Ballester R, Marchuk D, Boguski M, Saulino A, Letcher R Wigler M and Collins F (1990) The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell* **63**, 851-859.
8. Dasgupta B, Yi Y, Chen DY, Weber JD, and Gutmann DH (2005) Proteomic analysis reveals hyperactivation of the mTOR pathway in NF1-associated human and mouse brain tumors. *Cancer Research*, Advanced Online Publication
9. Feuerstein, N., and Mond, J. J. (1987) "Numatrin," a nuclear matrix protein associated with induction of proliferation in B lymphocytes. *Journal of Biological Chemistry* **262**, 11389-11397
10. Falini B, Mecucci C, Tiacci E, Acalay M, Rosati R, Pasqualucci L, Mandelli F, Lo-Coco F, Pelicci PG and MArtelli MF (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *The New England Journal of Medicine* **352**, 254-266.
11. Meyuhas O (2000) Synthesis of the translational apparatus is regulated at the translational level. *European Journal of Biochemistry* **267**, 6321-6330.
12. Brady, S.N., Yu, Y., Maggi, L.B., Weber, J.D. 2004. ARF impedes NPM/B23 shuttling in an Mdm2 sensitive tumor suppressor pathway. *Molecular and Cellular Biology* **24**, 9327-9338.
13. Bertwistle, D., M. Sugimoto, and C.J. Sherr. 2004. Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. *Molecular and Cellular Biology* **24**, 985-96.
14. Pelletier, C.L., L.B. Maggi, S.N. Brady, D.K. Scheidenhelm, D.H. Gutmann, and J.D. Weber. 2006. TSC1 sets the rate of ribosome nuclear export through translation of nucleophosmin. *Cancer Research*, in press.

APPENDICES

- "Nucleophosmin mediates mammalian target of rapamycin-dependent actin cytoskeleton dynamics and proliferation in neurofibromin-deficient astrocytes", Sandsmark et al., In press *Cancer Research*.

Research Article

Nucleophosmin mediates mammalian target of rapamycin-dependent actin cytoskeleton dynamics and proliferation in neurofibromin-deficient astrocytes

Danielle K. Sandsmark[1], Huabiao Zhang[1], Balazs Hegedus[1], Corey L. Pelletier[2], Jason D. Weber[2,3], and David H. Gutmann[1*]

From the Departments of [1]Neurology, [2]Molecular Oncology, and [3]Cell Biology, Washington University School of Medicine, St. Louis, MO 63110

Address correspondence to: David H. Gutmann, MD,PhD
Department of Neurology
Washington University School of Medicine
Box 8111; 660 S. Euclid Avenue
St. Louis, MO 63110
Phone 314-362-7379; FAX 314-362-2388
E-mail: gutmannd@wustl.edu

Running title: NPM regulates astrocyte proliferation and motility

Key words: glioma, neurofibromin, nucleophosmin, mTOR, astrocyte

ABSTRACT

Neurofibromatosis type 1 (NF1) is a common autosomal dominant tumor predisposition syndrome in which affected individuals develop astrocytic brain tumors (gliomas). To determine how the *NF1* gene product (neurofibromin) regulates astrocyte growth and motility relevant to glioma formation, we have employed *Nf1*-deficient primary murine astrocytes. *Nf1*-/- astrocytes exhibit increased protein translation and cell proliferation, which are mediated by Ras-dependent hyperactivation of the mammalian target of rapamycin (mTOR) protein, a serine/threonine protein kinase that regulates ribosomal biogenesis, protein translation, actin cytoskeleton dynamics, and cell proliferation. In this study, we demonstrate that *Nf1*-deficient astrocytes have fewer actin stress fibers and exhibit increased cell motility compared to wild-type astrocytes, which are rescued by pharmacologic and genetic mTOR inhibition. We further show that mTOR-dependent regulation of actin stress fiber formation, motility, and proliferation requires rapamycin-sensitive activation of the Rac1 GTPase, but not 4EBP1/S6K. *Nf1*-/- astrocytes also exhibit increased protein translation and ribosomal biogenesis through increased expression of the nucleophosmin (NPM) nuclear-cytoplasmic shuttling protein. We found that NPM expression in *Nf1*-/- astrocytes was blocked by rapamycin *in vitro* and *in vivo*, and that expression of a dominant-negative NPM mutant protein in *Nf1-/-* astrocytes rescued actin stress fiber formation and restored cell motility and proliferation to wild-type levels. Together, these data demonstrate that neurofibromin regulates actin cytoskeleton dynamics and cell proliferation through an mTOR/Rac1-dependent signaling pathway and identify NPM as a critical mTOR effector mediating these biologic properties in *Nf1*-deficient astrocytes.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant tumor predisposition syndromes, affecting approximately 1 in 3000 people worldwide. Individuals with NF1 develop tumors involving both the peripheral and central nervous systems. Neurofibromas, the benign Schwann cell tumor for which the disorder is named, are seen in 85-95% of adults with NF1 , whereas in children, the most common tumor is an astrocytic neoplasm (glioma) involving the optic pathway (1). Although NF1-associated OPGs typically exhibit low proliferative indices, they can be locally invasive and infiltrate into normal brain structures to result in significant morbidity.

The observation that individuals with NF1 are predisposed to develop astrocytic tumors suggests that the *NF1* gene product, neurofibromin, is a critical regulator of astrocyte growth. In this regard, neurofibromin functions as a Ras GTPase activating protein (GAP) to negatively regulate the Ras proto-oncogene by accelerating the conversion of Ras from a GTP-bound active form to a GDP-bound inactive form (2). Consistent with the role of neurofibromin as a Ras-GAP, loss of neurofibromin expression in human NF1-associated tumors as well as in cells derived from *Nf1*-deficient mice results in hyperactivation of Ras and downstream Ras targets, including mitogen activated protein kinase (MAPK), RAF, and Akt (3-6).

To study the role of neurofibromin in astrocyte growth regulation and glioma formation, our laboratory has developed several murine models of NF1-associated neurologic disease by conditionally inactivating *Nf1* expression in brain astrocytes (7-9). Studies of these genetically-engineered mouse models and derivative astrocytes have revealed that *Nf1-/-* astrocytes have several unique biologic properties. First, *Nf1-/-* astrocytes exhibit increased cell proliferation both *in vitro* and *in vivo* (8, 10). This increase in astrocyte proliferation requires Ras-dependent hyperactivation of the mammalian target of rapamycin (mTOR), a serine/threonine protein

kinase that regulates ribosomal biogenesis, protein translation, and cell growth/proliferation. In support of this finding, *Nf1-/-* fibroblasts and malignant peripheral nerve sheath tumors derived from NF1 patients exhibit Ras-dependent mTOR pathway hyperactivation (11), indicating that mTOR is an important downstream effector of neurofibromin signaling. Second, *Nf1-/-* primary astrocytes exhibit a two-fold increase in cell motility that is associated with hyperactivation of the small GTPase Rac1 (9). Similarly, *Nf1-/-* Schwann cells have enhanced chemokinetic and chemotactic migration compared to wild-type controls (12). Third, *Nf1-/-* astrocytes demonstrate an eight-fold increase in protein synthesis rates associated with increased expression of ribosomal proteins, including the nuclear-cytoplasmic shuttling protein nucleophosmin (NPM; 10). Consistent with a role for neurofibromin in regulating protein synthesis and ribosomal biogenesis, the increased protein translation rate in *Nf1-/-* astrocytes is completely rescued by treatment with the mTOR inhibitor rapamycin.

In this study, we sought to define the molecular signaling events that underlie the increases in cell proliferation and cell motility characteristic of *Nf1-/-* astrocytes. In addition to regulating astrocyte proliferation, we find that mTOR regulates actin stress fiber formation and astrocyte motility, indicating that mTOR is an important regulator of actin cytoskeleton dynamics in astrocytes. We demonstrate that Rac1 functions downstream of mTOR to regulate actin stress fiber formation, astrocyte motility, and astrocyte proliferation. We identify nucleophosmin (NPM) as a critical target of mTOR signaling both *in vitro* and *in vivo*, and show that mTOR/Rac1 signaling is required for the increased expression of NPM in *Nf1-/-* astrocytes *in vitro* and *in vivo*. Moreover, we demonstrate that NPM regulates *Nf1*-/- astrocyte actin stress fiber formation as well as *Nf1*-/- astrocyte motility and proliferation *in vitro*. Together, these data identify a unique, mTOR-dependent signaling pathway that regulates actin cytoskeleton

dynamics and cell proliferation in astrocytes and suggests that targeting these signaling intermediates may be useful for treating disorders characterized by mTOR pathway hyperactivation.

MATERIALS AND METHODS

Mice. *Nf1*$^{f/f}$ mice were generously provided by Dr. Luis Parada (University of Texas Southwestern, Dallas, TX) and maintained as continuous breeding colonies by intercrossing (13). To conditionally inactivate *Nf1* in astrocytes and to generate the *Nf1* optic glioma model (*Nf1*$^{f/mut}$;GFAP-Cre mice), *Nf1*$^{f/f}$ mice were crossed with mice expressing Cre recombinase under the control of the human 2.2kb GFAP promoter as previously described (7, 8). Mice were used in accordance with established animal studies protocols at the Washington University School of Medicine.

Primary astrocyte cultures. Murine neocortical astrocyte cultures were generated from postnatal day 2 *Nf1*$^{f/f}$ pups as described previously (9) and maintained in astrocyte growth media (DMEM containing 10% fetal bovine serum and antibiotics). This method generates cultures that consist of >97% GFAP-immunoreactive astrocytes. To inactivate the *Nf1* gene, *Nf1*$^{f/f}$ astrocytes (passage 1) were treated with adenovirus (Ad5) encoding Cre recombinase (University of Iowa Gene Transfer Vector core, Iowa City, IA). Wild-type astrocytes were treated identically with an adenovirus encoding LacZ. Four days after Cre adenovirus treatment, neurofibromin could no

longer be detected by Western blot (data not shown; (9). Astrocytes were analyzed at passage 2 (12-15 days after dissection).

Pharmacologic inhibitors. Rapamycin (Sigma, St. Louis, MO) was used at a concentration of 10 nM. Leptomycin B (Calbiochem, San Diego, CA) was used at a concentration of 20 ng/mL. All pharmacologic treatments were for 16-18 hours unless otherwise indicated.

Cell motility. Transwell Boyden chambers with 0.8 μM membranes (Costar, Corning, NY) were employed to measure astrocyte motility (9). Matrigel (BD Biosciences, Bedford, MA) was added to the inside of the transwell and allowed to solidify at room temperature. Astrocytes were plated in triplicate transwells on the opposite side of the membrane and allowed to adhere for 1 hour. The transwells were rinsed in PBS and maintained in astrocyte growth media. The astrocytes were allowed to migrate towards the Matrigel for 48 hours followed by fixation of the membranes in 100% methanol for 30 minutes at -20°C. The membranes were stained with hematoxylin, rinsed, and dried prior to mounting on a glass slide for counting. For each membrane (3 per condition), 3 representative areas were counted at 40X magnification. Each experiment was repeated three times with identical results.

Cell proliferation. Astrocytes were plated (60,000 cells/well) in 24 well dishes on glass coverslips and allowed to adhere for 24 hours. Astrocytes were rinsed twice in serum-free DMEM and maintained in serum-free DMEM for 24 hours before exposure to BrdU (Sigma; 10 μg/mL) or ^{3}H-thymidine (1 μCi/μl) for 16 hours. Astrocytes exposed to BrdU were fixed for immunocytochemistry using antibodies against BrdU (Abcam; 1:200) and GFAP (Sigma; 1:200).

^{3}H-thymidine incorporation was determined by scintillation counting as previously described (10).

Immunocytochemistry. Astrocytes (passage 2) were plated in triplicate on glass coverslips in 24 well plates (60,000 cells/well) in astrocyte growth media. After 24 hours, astrocytes were rinsed twice and incubated in serum-free media for 18-24 hours. Pharmacologic inhibitors were added as indicated. Astrocytes were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked in 10% goat serum/1% BSA in PBS at 37°C. To identify focal adhesions, astrocytes were incubated with a vinculin monoclonal antibody (1:2500; Sigma) in 1% BSA in PBS overnight at 4°C. Alexa568-conjugated anti-mouse IgG secondary antibody (1:1000; Molecular Probes, Carlsbad, CA) was used for detection. To identify the actin cytoskeleton, astrocytes were incubated with BODIPY-phalloidin or Phalloidin-Alexa568 (Molecular Probes) for 20 minutes in the dark. Nuclei were counterstained with DAPI. Representative photomicrographs were obtained using a fluorescence microscope (Nikon Eclipse TE300 inverted microscope, Japan) equipped with a digital camera (Optronics, Goleta, CA).

Western blotting. Astrocytes were serum-starved overnight, harvested by scraping in PBS, and lysed in NP40 lysis buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris pH 7.6, 1 mM DTT) with protease inhibitors. Western blotting was performed as previously described (14). All antibodies are from Cell Signaling Technology (Danvars, MA) and used at a 1:1000 dilution unless otherwise noted. Primary antibodies recognizing Rac1 (clone 23A8, Upstate Biotechnology, Charlottesville, VA; 1:500), phospho-S6 (#2215), S6 (#2217), phospho-4EBP1 (#9459), 4EBP1 (#9452), phospho-Akt (#1140), Akt (#9272), phospho-PKCα (#9375), PKCα

(sc-208; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500), mTOR (#2972), and nucleophosmin (Zymed, Carlsbad, CA). HRP-conjugated secondary antibodies were purchased from Cell Signaling and detection was performed by enhanced chemiluminescence (Pierce, Rockford, IL).

Rac1 activation assay. GTP-bound Rac1 was measured using the Rac activation kit from Upstate Biotechnology according to the manufacturer's instructions. Briefly, astrocytes were lysed and incubated with PAK1 PBD-conjugated agarose beads. An aliquot of the lysate was saved for Western blotting to ensure equal protein loading. Beads were washed in lysis buffer containing protease inhibitors, boiled in Laemmli buffer and separated on SDS-PAGE gels for Western blotting.

Retroviral constructs. The mTOR siRNA was generated using the siXpress Human H1 PCR Vector System (Mirus, Milwaukee, WI; (14). Briefly, double stranded siRNA expression cassettes were generated by PCR using H1 promoter and gene specific primers. Several gene-specific sequences were tested for their ability to block mTOR or Pten protein expression. In these experiments, we used an mTOR siRNA corresponding to nucleotides 139-152 of the *Mus musculus mTOR* gene (NCBI Accession # NM_02009; sequence 5'-CATCTAGCAACGTGAGCGTCCTGC-3'). A control expression cassette encoded the H1 promoter alone. The PCR expression cassettes were cloned into pCR2.1 (Invitrogen), digested with EcoRI, and cloned into the MSCV.IRES.GFP retrovirus.

Viral transduction. MSCV.IRES.GFP retroviruses were transfected into 293T packing cells with ψ helper DNA using Lipofectamine 2000 (Invitrogen) as previously described (14). Astrocytes were passaged 7-8 days after dissection, treated with adenovirus (LacZ or Cre), and then transduced with filtered supernatant from the 293T cells collected over a 36 hour period. Transduced astrocytes were evaluated by fluorescence microscopy (Nikon Eclipse TE300 inverted microscope, Japan). Typically, more than 90% of astrocytes exhibited GFP expression, indicating efficient viral transduction. Each experiment was performed 2-3 times with identical results.

In vivo rapamycin treatment and immunohistochemistry. Rapamycin (Calbiochem) was prepared in a vehicle containing 5.2% PEG-400/5.2% Tween-80. Two month old *Nf1*$^{f/mut}$*;GFAP*-Cre mice were treated with daily injections (5 days/week) of rapamycin (5 mg/kg) or vehicle alone in 100 µl total volume. After two weeks of treatment, the mice were perfused transcardially with 4% paraformaldehyde, and the optic nerves dissected for paraffin embedding and sectioning. Slides were deparaffinized in xylene and subjected to microwave antigen retrieval. After washing and blocking steps, brain sections were incubated overnight with P-S6 (1:200) or NPM antibodies (1:1000) followed by incubation with biotinylated secondary antibodies (1:200) at room temperature for 1-2 hours. Immunoreactivity was visualized with the Vectastain ABC System and 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). All sections were photographed with a digital camera (Optronics) attached to an inverted microscope (Nikon).

RESULTS

Increased cell proliferation and cell motility as well as decreased actin stress fiber formation in Nf1-/- astrocytes are mediated by mTOR.

Previous studies in our laboratory have demonstrated that *Nf1*-/- astrocytes exhibit a two-fold increase in cell proliferation that is restored to wild-type levels by treatment with the mTOR inhibitor rapamycin (Fig. 1A, 10). Under these conditions, there was no statistically significant effect of rapamycin treatment on wild-type astrocyte proliferation. In addition, *Nf1-/-* astrocytes exhibit a two-fold increase in cell motility and altered cell spreading, two processes that require proper organization of the actin cytoskeleton (8, 9, 15). Recently, mTOR has also been shown to modulate actin cytoskeleton dynamics (16, 17). Based on these observations, we sought to determine whether the increase in cell motility observed in *Nf1-/-* astrocytes was also mediated by mTOR. Using a Boyden chamber assay, the increase in *Nf1-/-* astrocyte motility was ameliorated by treatment with 10 nM rapamycin (Fig. 1B). As before, no effect of rapamycin on wild-type astrocytes was seen. These data indicate that both increased astrocyte proliferation and motility are mediated by mTOR-dependent signaling in *Nf1-/-* astrocytes.

To extend our findings to other cytoskeleton properties, we next examined stress fiber formation in *Nf1*-/- astrocytes. Actin stress fibers are bundles of filamentous actin that extend through the cytoplasm and interact with membrane integrins at specialized regions of the cell membrane called focal adhesions to anchor the cell to the extracellular matrix (18). Actin stress fibers have been used as a model system to study actin cytoskeleton dynamics. To directly visualize the actin cytoskeleton, we used fluorophore-labeled phalloidin, a fungal toxin that specifically binds actin. Whereas wild-type astrocytes exhibit numerous actin stress fibers, *Nf1-*

/- astrocytes have few stress fibers (Fig. 1C). In addition, we performed immunocytochemistry for vinculin, a protein localized to focal adhesions (19). Consistent with a decrease in actin stress fibers, vinculin-positive, membrane-localized focal adhesions were nearly absent in *Nf1-/-* astrocytes whereas wild-type astrocytes had numerous focal adhesions (Figure 1C).

This stress fiber assay provided us with a tractable system to examine the effects of rapamycin on actin cytoskeleton dynamics as a function of time of treatment. In these studies, we treated wild-type and *Nf1-/-* astrocytes with a low concentration of rapamycin (10 nM) for various lengths of time (1 hour-24 hours). We observed a dramatic increase in actin stress fibers and vinculin-positive focal adhesions in *Nf1-/-* astrocytes even with 1 hour of rapamycin treatment (Fig. 1C). In contrast, rapamycin had no effect on stress fiber formation or focal adhesions in wild-type astrocytes.

To confirm that the effect of rapamycin on actin stress fiber formation was mediated by mTOR, we introduced an mTOR-specific siRNA into wild-type and *Nf1-/-* astrocytes. Knockdown of mTOR expression was confirmed by Western blot (Fig. 1D). Similar to astrocytes treated with rapamycin, genetic inhibition of mTOR in *Nf1-/-* astrocytes restored actin stress fiber formation (Fig. 1D). Together, these data indicate that mTOR regulates actin cytoskeleton dynamics and cell proliferation in *Nf1*-/- astrocytes.

mTOR-dependent regulation of actin stress fiber formation occurs independently of 4EBP1 and S6K.

Because mTOR signaling underlies the hyperproliferation, increased cell motility, decreased actin stress fiber formation (Fig. 1), and increased protein translation (10) in *Nf1-/-* astrocytes, we next sought to determine which mTOR downstream effectors are responsible for

mediating these biological phenotypes. The best studied mTOR effectors are ribosomal S6 kinase (S6K) and elongation factor 4E binding protein 1 (4EBP1), proteins that regulate rates of protein translation. First, we examined the activation status of these effectors using phosphorylation-specific antibodies. While the target of S6 kinase, ribosomal S6, is robustly hyperactivated in *Nf1-/-* astrocytes, there was no change in 4EBP1 phosphorylation at Thr37/Thr46 (Fig. 2A), two sites required for 4EBP1 inactivation and translation initiation (20). These data suggest that 4EBP1 is not an important mediator of the mTOR-dependent abnormalities in cell proliferation and actin cytoskeleton dynamics in *Nf1-/-* astrocytes.

To directly examine the effect of S6K on actin stress fibers, we overexpressed human S6K in wild-type astrocytes. While S6K overexpression in wild-type astrocytes resulted in increased S6 activation (see Fig. 5A), we observed no changes in actin stress fiber formation (Fig. 2B). Similarly, rapamycin had no effect on actin cytoskeleton organization in S6K-expressing astrocytes. Taken together, these data suggest that rapamycin-sensitive, mTOR-dependent regulation of the astrocyte actin cytoskeleton occurs independently of the mTOR effectors 4EBP1 and S6K.

Rac1, but not PKCα is hyperactivated in Nf1-/- astrocytes.

Recently, protein kinase C α (PKCα) has been implicated in mTOR-dependent regulation of the actin cytoskeleton (17). PKCα is regulated by phosphorylation Thr500, Thr641, and Ser660 and exhibits no activity without phosphorylation of all sites (21). Using a commercially-available antibody specific for PKCα phosphorylated at Thr641, we observed no changes in the PKCα phosphorylation in *Nf1-/-* astrocytes compared to wild-type astrocytes (Fig. 3A). Furthermore, PKCα activation was not sensitive to rapamycin treatment in astrocytes.

The small GTPase Rac1 has also been shown to act downstream of mTOR and modulate actin stress fiber formation (16). Previous studies from our laboratory have shown that *Nf1-/-* astrocytes have increased levels of active, GTP-bound Rac1 (9). To determine whether Rac1 hyperactivation was mediated by mTOR signaling in astrocytes, we treated wild-type and *Nf1-/-* astrocytes with rapamycin (10 nM) and assayed Rac1 activation. In these experiments, we found that rapamycin blocked Rac1 hyperactivation in *Nf1-/-* astrocytes (Figure 3B), indicating that Rac1 acts downstream of mTOR.

To determine whether Rac1 activation is required for the cytoskeleton abnormalities observed in *Nf1-/-* astrocytes, we inhibited Rac1 signaling using a mutant form of Rac1 ($Rac1^{N17}$) that functions in a dominant-negative fashion to inhibit the activity of endogenous Rac1. We found that the expression of $Rac1^{N17}$ in *Nf1-/-*, but not wild-type, astrocytes resulted in a dramatic increase in actin stress fiber formation (Fig. 3C). Similarly, $Rac1^{N17}$ expression also restored the increased motility observed in *Nf1-/-* astrocytes to wild-type levels (Fig. 3C). Taken together, these data indicate that increased Rac1 activity resulting from rapamycin-sensitive mTOR hyperactivation underlies the actin cytoskeleton abnormalities observed in *Nf1-/-* astrocytes.

Rac1 has also been shown to regulate cell proliferation in a variety of cell types (22, 23) and *Rac1-/-* mouse embryonic fibroblasts exhibit both impaired migration and cell proliferation (24). To determine whether Rac1 regulates cell proliferation in *Nf1-/-* astrocytes, we expressed $Rac1^{N17}$ in wild-type and *Nf1-/-* astrocytes and examined BrdU incorporation. While expression of the dominant-negative Rac1 had little effect on wild-type astrocytes, it restored proliferation of *Nf1-/-* astrocytes to wild-type levels (Fig. 3D). Taken together, these data indicate that Rac1

is an important regulator of mTOR-dependent actin stress fiber formation, cell motility, and proliferation in astrocytes.

Expression of nucleophosmin is regulated by mTOR in Nf1-/- astrocytes

Previous studies in our laboratory demonstrated that nucleophosmin (NPM) was one of several proteins that is highly expressed in *Nf1-/-* astrocytes (10). In addition, NPM is overexpressed in a variety of neoplasms, including prostate, ovarian and colon cancers. Moreover, *Npm*-deficient fibroblasts exhibit decreased proliferation (25). NPM is a nuclear export protein that interacts with nucleolar components of newly synthesized ribosomes to facilitate their transport from the nucleolus/nucleus to the cytoplasm and increase the rates of protein synthesis (26). Expression of a NPM mutant that is unable to shuttle from the nucleus to the cytoplasm blocks ribosomal export (26). In addition, treatment with rapamycin inhibits NPM protein expression in mouse embryonic fibroblasts (27). In *Nf1*-/- astrocytes, we also found that mTOR inhibition by rapamycin dramatically reduced NPM expression in *Nf1*-/- astrocytes with no effect on wild-type astrocytes (Fig. 4A).

To determine whether mTOR also regulates NPM expression *in vivo*, we employed a mouse model of NF1-associated optic glioma ($Nf1^{f/mut}$;*GFAP*-Cre;7). For these experiments, we treated 2 month old $Nf1^{f/mut}$;*GFAP*-Cre with either rapamycin (5 mg/kg) or vehicle for 2 weeks (10 doses; n= 5 mice/group). We found that rapamycin treatment dramatically reduced mTOR pathway activation as evidenced by P-S6 immunohistochemistry (Fig. 4B). Moreover, NPM expression was also decreased by rapamycin treatment. Together, these data demonstrate that mTOR is a critical regulator of NPM expression in astrocytes both *in vitro* and *in vivo*.

Because S6K/S6 and Rac1 were the mTOR effectors activated in *Nf1-/-* astrocytes, we next sought to determine whether either of these mTOR targets regulated NPM expression. When S6K was overexpressed in wild-type astrocytes, there was no change in NPM expression, despite an increase in S6 phosphorylation (Fig. 5A). This is in stark contrast to NPM regulation in primary MEFs where S6K is a potent inducer of NPM protein expression (27) and highlights the potential differences in mTOR signaling mechanisms between fibroblasts and astrocytes. However, when $Rac1^{N17}$ was expressed in *Nf1-/-* astrocytes, NPM expression was attenuated. No change in S6 or Akt (data not shown) activity was observed following $Rac1^{N17}$ expression in *Nf1-/-* or wild-type astrocytes, again demonstrating that Rac1 functions downstream of mTOR. Collectively, these results suggest that NPM functions downstream of mTOR and Rac1 via an S6K-independent mechanism (Fig. 5B).

Nucleophosmin mediates alterations in actin cytoskeleton dynamics and cell proliferation in Nf1-/- astrocytes.

To determine whether NPM regulates actin stress fiber formation in *Nf1-/-* astrocytes, we used two complementary approaches to disrupt NPM function. One of the major roles of NPM is to mobilize ribosomes from the nucleolus to the cytosol (26). To disrupt this process, we expressed a mutant form of NPM (NPM double leucine mutant; NPMdL) that prevents NPM shuttling from the nucleolus to the cytoplasm. Previous studies from our laboratory have shown that this mutant behaves like a true dominant-negative protein, forming hetero-oligomers with endogenous NPM and blocking endogenous NPM from shuttling from the nucleus to the cytoplasm (26). Expression of the NPMdL mutant rescued actin stress fiber formation in *Nf1-/-* astrocytes, but had no effect on wild-type astrocytes (Fig. 6A). Second, we took advantage of

the observation that NPM export from the nucleolus/nucleus is dependent on its interaction with the nuclear export protein CRM1 (26). Leptomycin B is an antibiotic derived from *Streptomyces* that binds to CRM1 to specifically block CRM1-dependent nuclear export (28). When *Nf1-/-* astrocytes were treated with 20ng/mL leptomycin B, actin stress fiber formation was rescued (Fig. 6A). As before, no effect of leptomycin B treatment on wild-type astrocytes was observed. Next, we directly assessed the effect of increased NPM expression on actin stress fiber formation in wild-type astrocytes. In these experiments, increased expression of His-tagged NPM resulted in decreased actin stress fiber formation in wild-type astrocytes which was rescued by leptomycin B treatment (Fig. 6B), similar to *Nf1*-/- astrocytes. Together, these data demonstrate that increased expression of functionally mobile NPM protein mediates the decrease in actin stress fibers characteristic of *Nf1-/-* astrocytes.

To extend these observations, we next examined the effect of inhibiting NPM function on *Nf1*-/- astrocyte motility and proliferation. Similar to what we observed for rapamycin treatment and $Rac1^{N17}$ expression, expression of the NPMdL mutant restored cell motility (Fig. 6C) and proliferation (Fig. 6D) to wild-type levels. Together, these data indicate that NPM is a critical mTOR effector important for regulating actin cytoskeleton dynamics and cell proliferation in *Nf1*-/- astrocytes.

DISCUSSION

mTOR has been shown to integrate signals from a variety of extracellular inputs, including growth factors, amino acids, glucose, ATP, and oxygen. In addition, mTOR-dependent signaling modulates numerous cellular properties, including cell proliferation, cell motility, and protein translation. We had previously found that *Nf1-/-* astrocytes exhibit defects

in each of these properties, suggesting that *Nf1*-deficient astrocytes would represent a good model system to examine the mechanisms underlying mTOR regulation of cell proliferation and motility. In this study, we use both pharmacologic and genetic approaches to demonstrate that the *Nf1* gene product neurofibromin regulates actin stress fiber formation, astrocyte motility, and astrocyte proliferation in an mTOR-dependent fashion. Using *Nf1*-/- astrocytes, we further show that neurofibromin regulation of these important biological properties are mediated by Rac1-dependent modulation of NPM expression and function.

The ability of mTOR to regulate these diverse biologic properties is not unique to neurofibromin. Studies on two other genetic disorders that predispose individuals to glioma formation have revealed roles for mTOR pathway regulation of cell proliferation and motility. Inactivation of the tumor suppressor gene *PTEN,* a negative regulator of phoshotidylinositol-3-kinase (PI3K) signaling, causes Lhermitte-Duclos disease (29) and *PTEN* inactivation is the one of the most common genetic changes observed in human gliomas (30). When *Pten* is inactivated in astrocytes, astrocyte proliferation is increased *in vitro* and *in vivo* (31). Moreover, in a murine glioma model, *Pten* inactivation promotes astrocyte invasion *in vitro* and *in vivo* (32). Similarly, tuberous sclerosis complex (TSC) is caused by mutations in one of two genes, *TSC1* or *TSC2,* that negatively regulate mTOR signaling (33). Inactivation of either *Tsc1* or *Tsc2* results in mTOR pathway hyperactivation *in vitro* and *in vivo* (34, 35) and inactivation of the *Tsc1/2* complex promotes increased cell proliferation (36-38). Moreover, loss of *Tsc1* results in dramatic increases in NPM protein expression and NPM-dependent increases in ribosomal biogenesis (27). The *Tsc1/2* gene products also regulate actin cytoskeleton dynamics. *Tsc1/2* inactivation results in a decrease in actin stress fibers (39) while *TSC2* re-expression in *TSC2*-/- human cells promotes increased cell adhesion and decreased cell migration (40). In addition,

Tsc1 inactivation results in decreased focal adhesions, while *Tsc1* overexpression promotes an increase in actin stress fibers and associated focal adhesions (41). Consistent with these findings, we have inactivated *Pten* and *Tsc1* in primary astrocytes and, like *Nf1* inactivation, these astrocytes exhibit fewer actin stress fibers that can be rescued by short-term rapamycin treatment (D.K.S. and D.H.G., unpublished observations). Together, these data indicate that mTOR has a central role in the regulation of actin stress fiber formation, cell motility, and cell proliferation in astrocytes.

mTOR signals through two distinct protein complexes, one that is sensitive to rapamycin and one that is insensitive to rapamycin-mediated inhibition (16, 17). Based on our observations that actin stress fiber formation in *Nf1-/-* astrocytes was rescued by short-term rapamycin treatment, we first examined mTOR signaling intermediates that are known to be sensitive to rapamycin, 4EBP1 and S6K. However, our data show that 4EBP1 activation is not altered by *Nf1* inactivation and overexpression of S6K does not alter actin stress fiber formation in astrocytes. These results suggest that neurofibromin regulation of mTOR signaling operates in a rapamycin-sensitive manner involving effectors other than S6K and 4EBP1. Consistent with our findings in astrocytes, S6K1/2 inactivation in mice impairs viability, but does not block cell proliferation (42). Moreover, cell cycle progression in *S6K1/2-/-* mice remained sensitive to rapamycin. In addition, *Pten* inactivation in neurons results in an mTOR-dependent increase in neuronal soma size, which occurs independent of S6K1/2 signaling (43).

In *Nf1-/-* astrocytes, we found that another mTOR target, the small GTPase Rac1, acts downstream of mTOR to regulate actin stress fiber formation, cell motility, and cell proliferation. We provide several lines of evidence that the small GTPase Rac1 acts downstream of mTOR to regulate mTOR-dependent cellular processes. First, Rac1 hyperactivation in *Nf1-/-* astrocytes is

robustly blocked by rapamycin treatment. Second, genetic inhibition of Rac1 in *Nf1-/-* astrocytes using the $Rac1^{N17}$ mutant fails to block phosphorylation of the mTOR target ribosomal S6. Third, while mTOR can initiate a positive feedback loop to activate the upstream kinase Akt, we observed no changes in Akt activation following either short or long-term rapamycin treatment in *Nf1-/-* astrocytes (D.K.S. and D.H.G., unpublished observations). These data indicate that Rac1 activation in *Nf1*-/- astrocytes is the direct result of mTOR and not compensatory Akt activation. Finally, we demonstrate that genetic inhibition of Rac1 rescues actin stress fiber formation in *Nf1*-/- astrocytes, and restores *Nf1-/-* astrocyte motility and proliferation to wild-type levels. Together, these results indicate that Rac1 functions as an mTOR pathway signaling intermediate that regulates actin cytoskeleton dynamics and cell proliferation in *Nf1-/-* astrocytes.

One clue as to how mTOR/Rac1 hyperactivation might regulate astrocyte proliferation and motility derived from the observation that neurofibromin also regulates protein translation in a rapamycin-dependent mechanism (10). Protein translation requires the synthesis of new ribosomes, a process that requires both rRNA transcription and processing as well as active shuttling of ribosomal proteins and RNAs from the nucleus to the cytoplasm. A key regulator of ribosome nuclear export is NPM, which functions as a nuclear-cytoplasmic shuttling protein. Recent studies have shown that NPM directly interacts with ribosomal subunits to facilitate their nuclear export (26). In this fashion, NPM promotes an increase in the cytoplasmic pool of actively translating ribosomes and increases protein synthesis rates. We show that NPM is overexpressed in *Nf1-/-* astrocytes *in vitro* and in a *Nf1*murine optic glioma model *in vivo.* This is consistent with previous studies demonstrating that NPM expression is induced by mitogenic signals (44, 45). Furthermore, we demonstrate that NPM expression is regulated by mTOR, as mTOR inhibition by rapamycin inhibits NPM expression both *in vitro* and *in vivo*. This supports

previous studies demonstrating that NPM is highly expressed in *Tsc1-/-* fibroblasts and is blocked by rapamycin treatment (27). This increase in NPM expression is due to increased translation of existing NPM transcripts, as rapamycin treatment or *Tsc1* re-expression suppresses NPM translation (27).

Our data now show that the ability of NPM to shuttle between the nucleus and cytoplasm is a critical step in NPM-dependent regulation of actin cytoskeleton dynamics and proliferation, as inhibition of NPM shuttling using both genetic (NPMdL mutant) and pharmacologic (leptomycin B) inhibitors rescues these phenotypes in *Nf1-/-* astrocytes. The exact mechanism by which NPM regulates cell motility and proliferation in astrocytes is currently unclear. It is possible that NPM-mediated increases in cytosolic ribosomes could lead to the production of specific proteins involved in cell motility and proliferation. In this regard, NPM associates with mature ribosomes and actively translating polysomes in the cytosol (26), suggesting that it has cytosolic functions at the polysome. At the polysome, NPM could regulate the translation of specific mRNA transcripts by the polysome machinery or assist in this process by supplying more cytosolic ribosomes available for translation. Support for this motion derives from a previous study which demonstrated that mitogenic signaling by Akt or Ras has distinct effects on the recruitment of specific mRNA transcripts to polysomes (46). When Akt or Ras signaling was blocked by acute pharmacologic inhibition, there was little change in mRNA transcription but a dramatic change in the profiles of polysome-associated mRNAs. Future studies examining the profiles of actively translated proteins in wild-type and *Nf1-/-* astrocytes may elucidate the mechanism underlying NPM regulation of actin stress fiber formation, motility, and proliferation in astrocytes.

Together, these studies indicate that neurofibromin/mTOR signaling regulates actin cytoskeleton dynamics and cell proliferation in astrocytes and identifies Rac1 and NPM as components of the mTOR signaling pathway. This study suggests that therapies that specifically target these signaling molecules may prove useful for the treatment of NF1 and related disorders characterized by mTOR pathway activation. As gliomas frequently exhibit mTOR pathway activation (47) and NPM is highly expressed in human NF1-associated gliomas (D.H.G., unpublished observations), future studies aimed at understanding the molecular mechanisms that govern mTOR/Rac1/NPM regulation of astrocyte biology may lead to improved treatments for these deadly cancers.

Acknowledgements. We would like to thank Ryan Emnett, Nisha Wadhwani, and Diane Ma for their excellent technical assistance. This work was supported by grants from the Department of Defense and National Cancer Institute to D.H.G and the Pew Scholars Program in Biomedical and Department of Defense (W81XWH0610129) to JDW.

REFERENCES

1. Listernick R, Charrow J, Gutmann DH. Intracranial gliomas in neurofibromatosis type 1. Am J Hum Genet 1999;89:38-44.
2. DeClue JE, Papageorge AG, Fletcher JA, et al. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell 1992;69:265-73.
3. Basu TN, Gutmann DH, Fletcher JA, et al. Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients. Nature 1992;356:713-5.
4. Bollag G, Clapp DW, Shih S, et al. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nat Genet 1996;12:144-8.
5. Largaespada DA, Brannan CI, Jenkins NA, Copeland NG. Nf1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukaemia. Nat Genet 1996;12:137-43.
6. Sherman LS, Atit R, Rosenbaum T, Cox AD, Ratner N. Single cell Ras-GTP analysis reveals altered Ras activity in a subpopulation of neurofibroma schwann cells but not fibroblasts. J Biol Chem 2000;275:30740-5.
7. Bajenaru ML, Hernandez MR, Perry A, et al. Optic nerve glioma in mice requires astrocyte Nf1 gene inactivation and Nf1 brain heterozygosity. Cancer Res 2003;63:8573-7.
8. Bajenaru ML, Zhu Y, Hedrick NM, et al. Astrocyte-specific inactivation of the neurofibromatosis 1 gene (NF1) is insufficient for astrocytoma formation. Mol Cell Biol 2002;22:5100-13.

9. Dasgupta B, Li W, Perry A, Gutmann DH. Glioma formation in neurofibromatosis 1 reflects preferential activation of K-RAS in astrocytes. Cancer Res 2005;65:236-45.

10. Dasgupta B, Yi Y, Chen DY, Weber JD, Gutmann DH. Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in Neurofibromatosis 1-associated human and mouse brain tumors. Cancer Res 2005;65:2755-60.

11. Johannessen CM, Reczek EE, James MF, et al. The NF1 tumor suppressor critically regulates TSC2 and mTOR. Proc Natl Acad Sci USA 2005;102:8573-8.

12. Huang Y, Rangwala F, Fulkerson PC, et al. Role of TC21/R-Ras2 in enhanced migration of neurofibromin-deficient Schwann cells. Oncogene 2004;23:368-78.

13. Zhu Y, Romero MI, Ghosh P, et al. Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. Genes Dev 2001;15:859-76.

14. Uhlmann EJ, Li W, Scheidenhelm DK, et al. Loss of tuberous sclerosis complex 1 (Tsc1) expression results in increased Rheb/S6K pathway signaling important for astrocyte cell size regulation. GLIA 2004;47:180-8.

15. Bajenaru ML, Donahoe J, Corral T, et al. Neurofibromatosis 1 (NF1) heterozygosity results in a cell-autonomous growth advantage for astrocytes. GLIA 2001;33:314-23.

16. Jacinto E, Loewith R, Schmidt A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol 2004;6:1122-8.

17. Sarbassov DD, Ali SM, Kim D-H, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 2004;14:1296-302.

18. Sechler JL, Corbett SA, Wenk MB, Schwarzbauer JE. Modulation of cell-extracellular matrix interactions. Ann NY Acad Sci 1998;857:143-54.

19. Geiger B, Tokuyasu KT, Dutton AH, Singer SJ. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. Proc Natl Acad Sci USA 1980;77:4127-31.

20. Gingras A-C, Gygi SP, Raught B, et al. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev. 1999;13:1422-37.

21. Nakashima S. Protein kinase C alpha: regulation and biological function. J Biochem 2002;132:669-75.

22. Yang C, Liu Y, Leskow FC, Weaver VM, Kazanietz MG. Rac-GAP-dependent inhibition of breast cancer cell proliferation by beta2-chimerin. J Biol Chem 2005;280:24363-70.

23. Wang G, Beier F. Rac1/Cdc42 and RhoA GTPases antagonistically regulate chondrocyte proliferation, hypertrophy, and apoptosis. J Bone Miner Res 2005;20:1022-31.

24. Vidali L, Chen F, Cicchetti G, Ohta Y, Kwiatkowski DJ. Rac1-null mouse embryonic fibroblasts are motile and respond to platelet-derived growth factor. Mol Biol Cell 2006;17:2377-90.

25. Grisendi S, Bernardi R, Rossi M, et al. Role of nucleophosmin in embryonic development and tumorigenesis. Nature 2005;437:147-53.

26. Yu Y, Maggi LB, Jr., Brady SN, et al. Nucleophosmin is essential for ribosomal protein L5 nuclear export Mol Cell Biol 2006;26:3798-809.

27. Pelletier CL, Maggi LB, Jr., Scheidenhelm DK, Gutmann DH, Weber JD. *Tsc1* sets the rate of ribosome export and protein synthesis through nucleophosmin translation. Cancer Res; in press.

28. Kudo N, Matsumori N, Taoka H, et al. Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proc Natl Acad Sci USA 1999;96:9112-7.

29. Nelen MR, Padberg GW, Peeters EAJ, et al. Localization of the gene for Cowden disease to chromosome 10q22-23. Nat Genet 1996;13:114-6.

30. Ohgaki H, Dessen P, Jourde B, et al. Genetic pathways to glioblastoma: a population-based study. Cancer Res 2004;64:6892-9.

31. Fraser MM, Zhu X, Kwon CH, et al. Pten loss causes hypertrophy and increased proliferation of astrocytes in vivo. Cancer Res 2004;64:7773-9.

32. Xiao A, Yin C, Yang C, et al. Somatic induction of Pten loss in a preclinical astrocytoma model reveals major roles in disease progression and avenues for target discovery and validation. Cancer Research 2005;65:5172-80.

33. Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J. Rheb binds and regulates the mTOR kinase. Curr Biol 2005;15:702-13.

34. Inoki K, Li Y, Zhu T, Wu J, Guan K-L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 2002;4:648-57.

35. Kwiatkowski DJ, Zhang H, Bandura JL, et al. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in *Tsc1* null cells. Hum Mol Genet 2002;11:525-34.

36. Benvenuto G, Li S, Brown SJ, et al. The tuberous sclerosis-1 (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination. Oncogene 2000;19:6306-16.

37. Miloloza A, Rosner M, Nellist M, et al. The TSC1 gene product, hamartin, negatively regulates cell proliferation. Hum Mol Genet 2000;9:1721-7.

38. Soucek T, Rosner M, Miloloza A, et al. Tuberous sclerosis causing mutants of the TSC2 gene product affect proliferation and p27 expression. Oncogene 2001;20:4904-9.

39. Goncharova E, Goncharov D, Noonan D, Krymskaya VP. TSC2 modulates actin cytoskeleton and focal adhesion through TSC1-binding domain and the Rac1 GTPase. J Cell Biol 2004;167:1171-82.

40. Astrinidis A, Cash TP, Hunter DS, et al. Tuberin, the tuberous sclerosis complex 2 tumor suppressor gene product, regulates Rho activation, cell adhesion, and migration. Oncogene 2002;21:8470-6.

41. Lamb RF, Roy C, Diefenbach TJ, et al. The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho. Nat Cell Biol 2000;2:281-7.

42. Pende M, Um SH, Mieulet V, et al. S6K1-/-/S6K2-/- mice exhibit perinatal lethality and rapamycin-sensitive 5' terminal oligopyrimdine mTOR translation and reveal a mitogen activated protein kinase-dependent S6 kinase pathway. Mol Cell Biol 2004;24:3112-24.

43. Chalhoub N, Kozma SC, Baker SJ. S6k1 is not required for Pten-deficient neuronal hypertrophy. Brain Res 2006;1100:32-41.

44. Feuerstein N, Spiegel S, Mond JJ. The nuclear matrix protein, numatrin (B23), is associated with growth factor-induced mitogenesis in Swiss 3T3 fibroblasts and with T lymphocyte proliferation stimulated by lectins and anti-T cell antigen receptor antibody. J Cell Biol 1988;107:1629-42.

45. Feuerstein N, Chan PK, Mond JJ. Identification of numatrin, the nuclear matrix protein associated with induction of mitogenesis, as the nucleolar protein B23. Implication for

the role of the nucleolus in early transduction of mitogenic signals. J Biol Chem 1988;263:10608-12.

46. Rajasekhar VK, Viale A, Socci ND, et al. Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. Mol Cell 2003;12:889-901.

47. Riemenschneider MJ, Betensky RA, Pasedag SM, Louis DN. Akt ctivation in human glioblastomas enhances proliferation via TSC2 and S6 kinase signaling. Cancer Res 2006;66:5618-23.

FIGURE LEGENDS

Figure 1. *Nf1*-/- astrocytes exhibit mTOR-dependent increases in cell proliferation and cell motility, as well as decreased actin stress fiber formation. (A) *Nf1-/-* astrocytes exhibit a two-fold increase in cell proliferation as determined by ^{3}H-thymidine incorporation which is reduced to wild-type levels by treatment with 10 nM rapamycin. (B) *Nf1*-/- astrocytes exhibit nearly a two-fold increase in cell migration using a Boyden chamber assay, which is reduced to wild-type levels by treatment with 10 nM rapamycin. (C) Serum-starved *Nf1*-/- astrocytes treated with 10 nM rapamycin (rapa) for 1 or 24 hours exhibit a dramatic increase in stress fiber formation (phalloidin) and focal adhesions (vinculin) as determined by immunocytochemistry. (D) Expression of an mTOR-specific siRNA rescues actin stress fiber formation in *Nf1-/-* astrocytes. Western blotting of *Nf1-/-* astrocytes confirmed that the mTOR siRNA inhibited mTOR protein expression and pathway activation, as demonstrated by immunoblotting for phosphorylated S6 (P-S6). Total S6 serves as a protein loading control. Scale bar= 200 μm (20X). *, $p< 0.05$

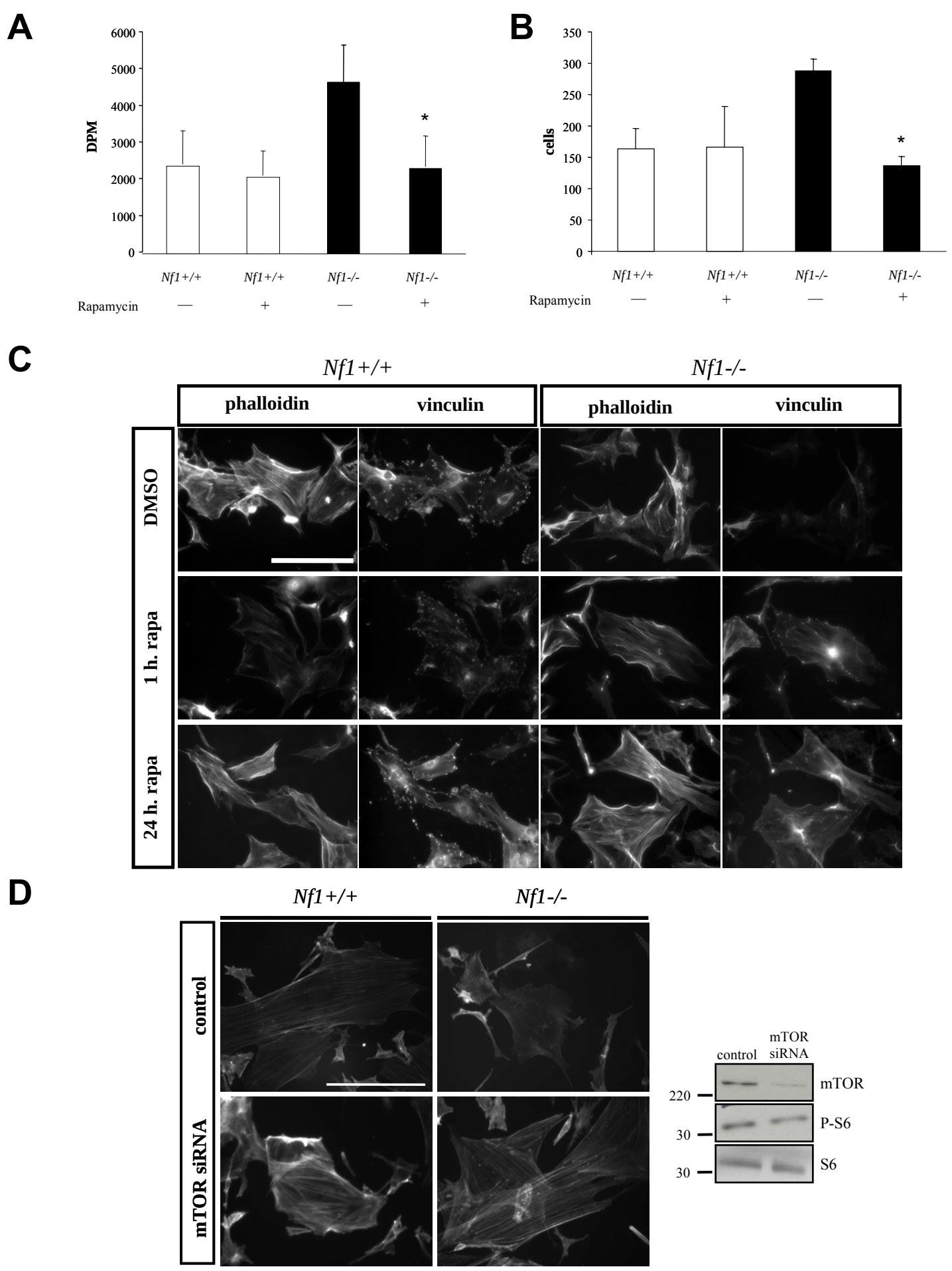
Figure 2. Decreased stress fiber formation in *Nf1*-/- astrocytes occurs independent of 4EBP1/S6K signaling. (A) 4EBP1 phosphorylation (Thr37/46) is similar in wild-type and *Nf1-/-* astrocytes while S6 phosphorylation (Ser240/244) is increased in *Nf1-/-* astrocytes. Lysates from wild-type (*Nf1*+/+) and *Nf1-/-* astrocytes were run on the same gel in non-consecutive lanes (black line). (B) Overexpression of S6K in wild-type astrocytes did not alter actin stress fiber formation in wild-type or *Nf1*-/- astrocytes using phalloidin cytochemistry. Scale bar= 200 μm (20X). *, $p< 0.05$

Figure 3. mTOR-dependent Rac1 hyperactivation is required for decreased actin stress fiber formation in *Nf1*-/- astrocytes. (A) The expression of activated PKCα (Thr638/641) is similar in wild-type and *Nf1-/-* astrocytes. Immunoblotting for total PKCα serves as a protein loading control. (B) GTP-bound Rac1 was immunoprecipitated from wild-type and *Nf1-/-* astrocytes treated with DMSO vehicle or 10 nM rapamycin using PAK1-PBD affinity chromatography. Equal protein loading was confirmed by immunoblotting for total Rac1 from a lysate aliquot prior to precipitation. GTP-bound Rac1 was increased in *Nf1*-/- astrocytes. Rac1 activation and S6 phosphorylation, but not total protein (Rac1, S6) expression, are blocked by rapamycin treatment. (C) Expression of a dominant-negative Rac1 mutant ($Rac1^{N17}$) restores actin stress fiber formation (phalloidin) and cell motility in *Nf1-/-* astrocytes to wild-type levels using phalloidin immunochemistry and Boyden chamber motility assay, respectively. (D) Expression of $Rac1^{N17}$ in *Nf1-/-* astrocytes decreases cell proliferation, as determined by BrdU incorporation. Scale bar= 200 μm (20X). *, $p< 0.05$

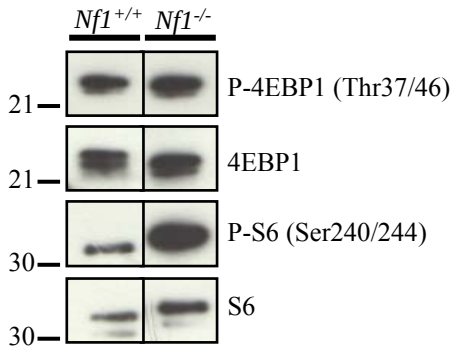
Figure 4. Nucleophosmin expression is regulated by mTOR signaling *in vitro* and *in vivo*. (A) Nucleophosmin (NPM) expression is increased in *Nf1-/-* astrocytes. Expression of NPM and P-S6 is inhibited by 10 nM rapamycin. Immunoblotting for total S6 demonstrates equal protein loading. (B) P-S6 and NPM are expressed at low levels in wild-type murine optic nerve ($Nf1^{f/f}$), but are dramatically increased in a murine model of optic glioma ($Nf1^{f/mut}$;*GFAP*-Cre), as shown by immunohistochemistry. Following rapamycin treatment *in vivo*, both NPM and P-S6 expression are decreased in the mouse optic gliomas. Scale bar=200 μm (10X).

Figure 5. NPM expression is regulated by Rac1^{N17}, but not S6K, in *Nf1-/-* astrocytes. (A) Overexpression of the mTOR downstream target S6K in wild-type astrocytes did not result in an increase in NPM expression. Phosphorylation of the S6K target ribosomal S6, but not total S6 protein, was increased in astrocytes overexpressing S6K. (B) Expression of Rac1^{N17} in *Nf1-/-* astrocytes decreases NPM expression, but does not attenuate S6 phosphorylation.

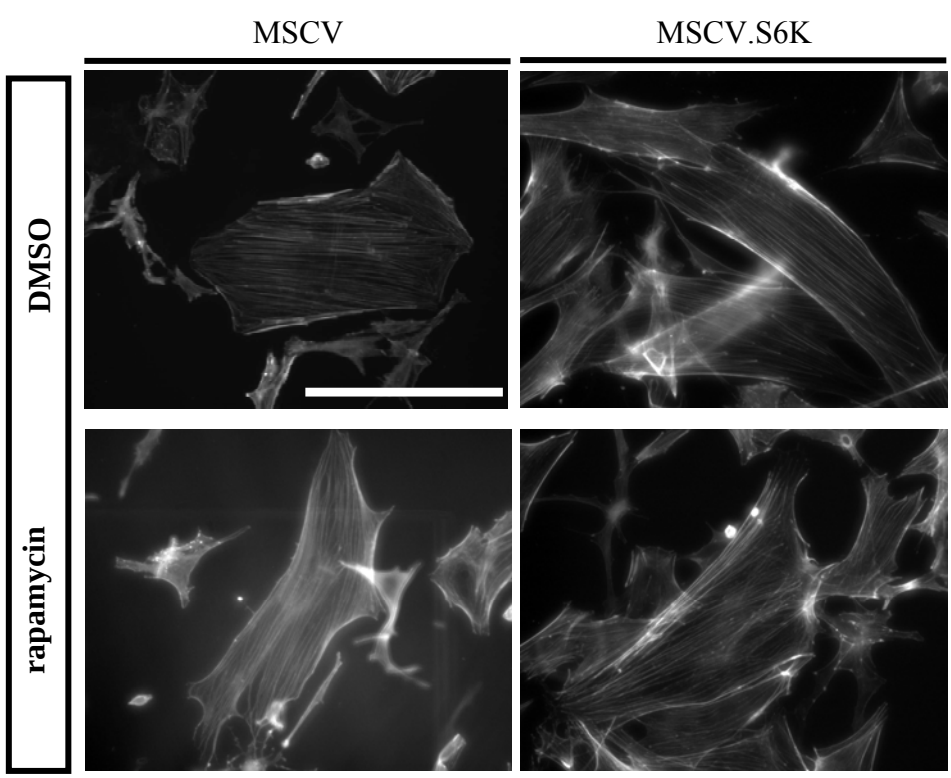
Figure 6. NPM mediates actin stress fiber formation, cell motility, and cell proliferation in *Nf1-/-* astrocytes. (A) NPM function was inhibited genetically using a mutant protein (NPMdL) that can not shuttle between the nucleus and cytoplasm, and pharmacologically using leptomycin B, an inhibitor of CRM1-dependent nuclear export. Inhibition of NPM shuttling activity either genetically or pharmacologically rescued actin stress fiber formation in *Nf1-/-* astrocytes, but did not alter actin stress fibers in wild-type cells. (B) Overexpression of NPM (MSCV.His.NPM) in wild-type astrocytes resulted in decreased actin stress fiber formation that was rescued by leptomycin B. Expression of the NPM shuttling mutant NPMdL in *Nf1-/-* astrocytes restored cell motility (C) and cell proliferation (D) to wild-type levels. Inhibition of NPM shuttling function had no effect on cell motility or proliferation in wild-type cells. Scale bar= 200 μm (20X). *, $p < 0.05$

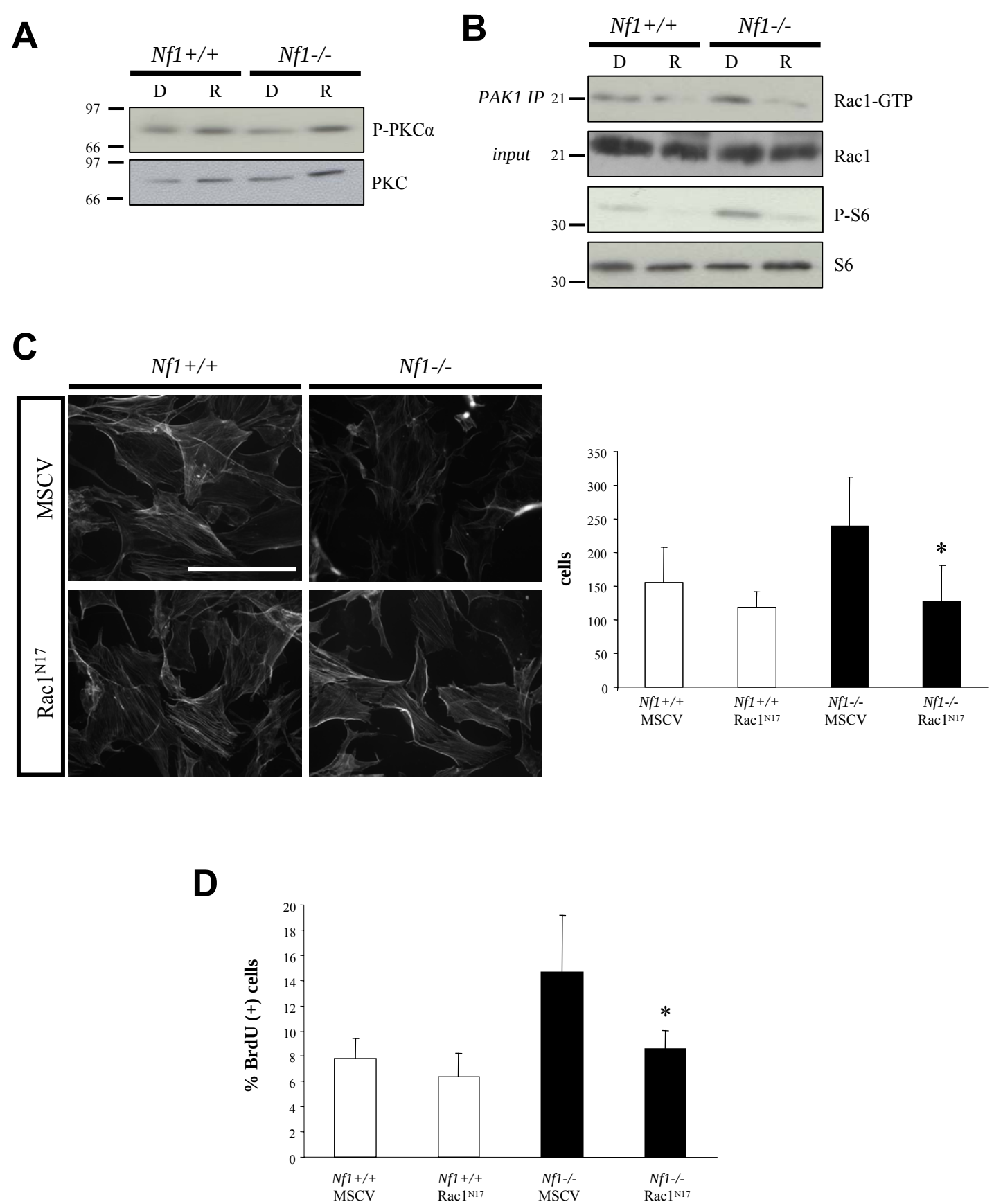

A
DPM
6000
5000
4000
3000
2000
1000
0
*
Nf1+/+
Nf1+/+
Nf1-/-
Nf1-/-
Rapamycin
—
+
—
+
B
cells
350
300
250
200
150
100
50
0
*
Nf1+/+
Nf1+/+
Nf1-/-
Nf1-/-
Rapamycin
—
+
—
+
C
Nf1+/+
Nf1-/-
phalloidin
vinculin
phalloidin
vinculin
DMSO
1 h. rapa
24 h. rapa
D
Nf1+/+
Nf1-/-
control
mTOR siRNA
control
mTOR siRNA
220
30
30
mTOR
P-S6
S6

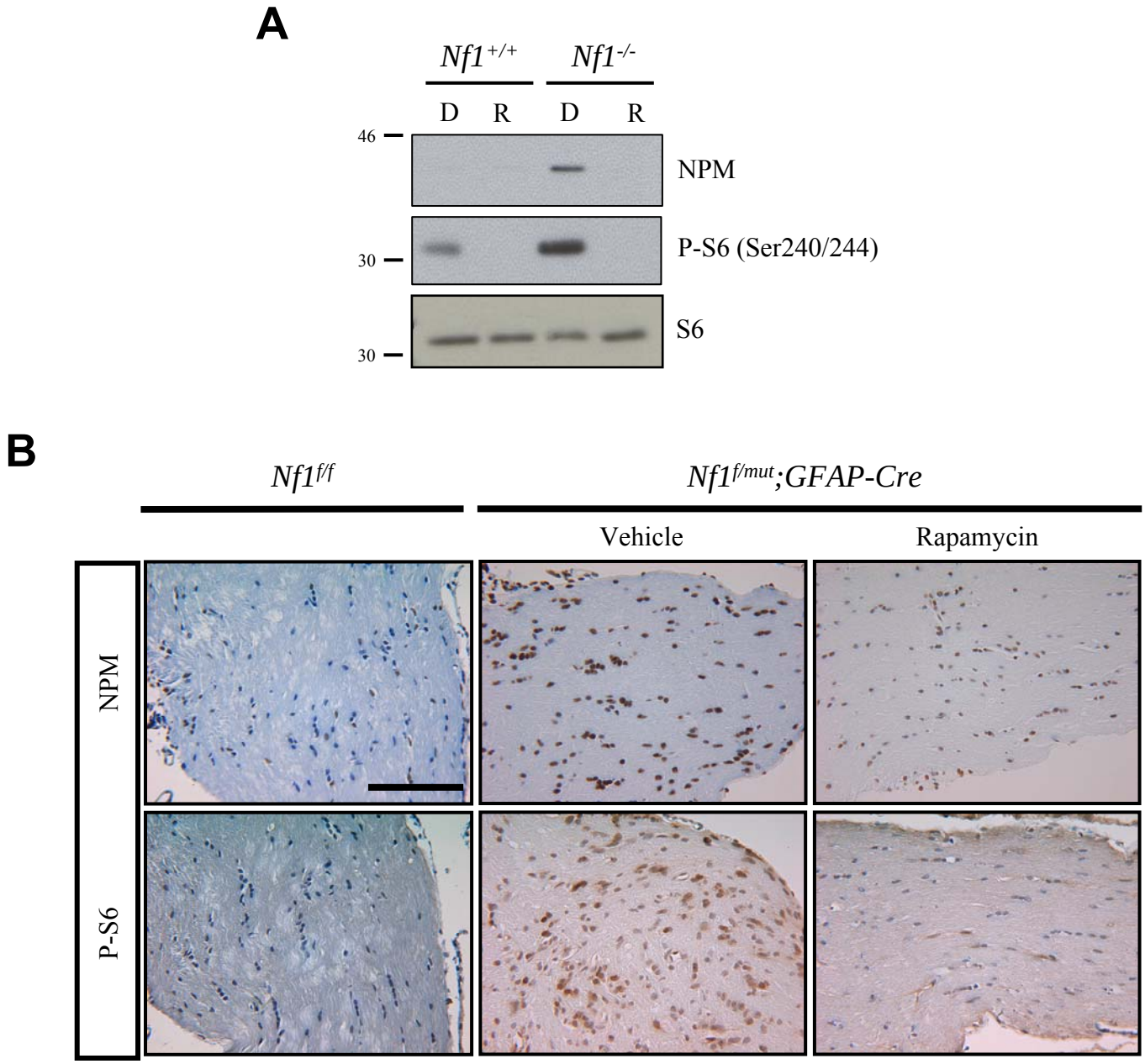
A

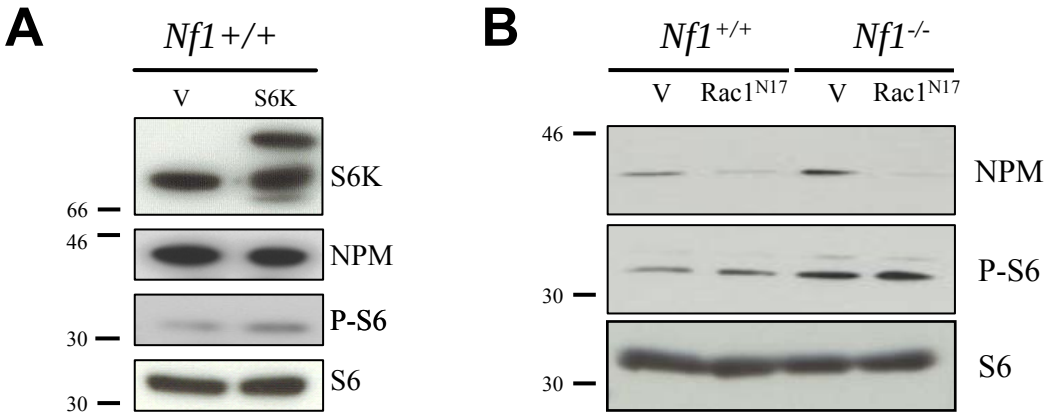

Nf1+/+
Nf1-/-
21
21
30
30
P-4EBP1 (Thr37/46)
4EBP1
P-S6 (Ser240/244)
S6

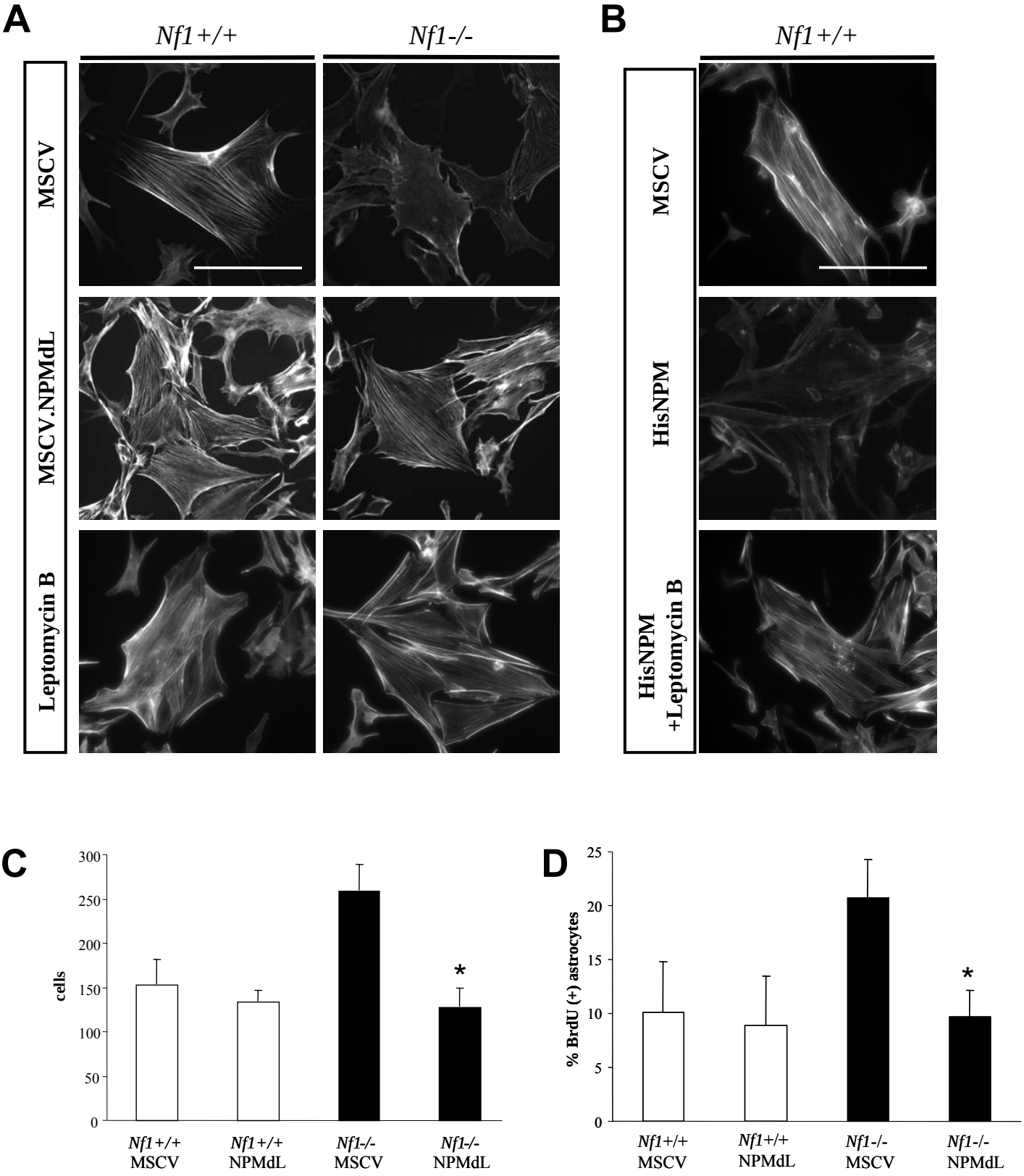
B


MSCV
MSCV.S6K
DMSO
rapamycin


A
Nf1+/+
Nf1-/-
D
R
D
R
97
66
97
66
P-PKCα
PKC
B
Nf1+/+
Nf1-/-
D
R
D
R
PAK1 IP
21
Rac1-GTP
input
21
Rac1
30
P-S6
30
S6
C
Nf1+/+
Nf1-/-
MSCV
Rac1N17
cells
350
300
250
200
150
100
50
0
*
Nf1+/+ MSCV
Nf1+/+ Rac1N17
Nf1-/- MSCV
Nf1-/- Rac1N17
D
% BrdU (+) cells
20
18
16
14
12
10
8
6
4
2
0
*
Nf1+/+ MSCV
Nf1+/+ Rac1N17
Nf1-/- MSCV
Nf1-/- Rac1N17


A
Nf1+/+
Nf1-/-
D
R
D
R
46
30
30
NPM
P-S6 (Ser240/244)
S6
B
Nf1f/f
Nf1f/mut;GFAP-Cre
Vehicle
Rapamycin
NPM
P-S6


A
Nf1+/+
V
S6K
66
46
30
30
S6K
NPM
P-S6
S6
B
Nf1+/+
Nf1-/-
V
Rac1N17
V
Rac1N17
46
30
30
NPM
P-S6
S6


A
Nf1+/+
Nf1-/-
MSCV
MSCV.NPMdL
Leptomycin B
B
Nf1+/+
MSCV
HisNPM
HisNPM
+Leptomycin B
C
cells
300
250
200
150
100
50
0
Nf1+/+
MSCV
Nf1+/+
NPMdL
Nf1-/-
MSCV
Nf1-/-
NPMdL
*
D
% BrdU (+) astrocytes
25
20
15
10
5
0
Nf1+/+
MSCV
Nf1+/+
NPMdL
Nf1-/-
MSCV
Nf1-/-
NPMdL
*